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Inhibition of pigeon breast muscle α-ketoglutarate dehydrogenase by phosphonate analogues of α-ketoglutarate

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Succinylphosphonate (SP) is a powerful inhibitor of α-ketoglutarate dehydrogenase (KGD). Methylation of the phosphonate reduces its inhibitory effect. The complex of KGD with SP undergoes a kinetically slow transition similar to the process observed during catalysis. α-Ketoglutarate binds to the enzyme–inhibitor complex, preventing its isomerisation.

α-Ketoglutarate dehydrogenase; Transition state analogue; Succinylphosphonate; Slow isomerisation

1. INTRODUCTION

α-Ketoglutarate dehydrogenase (KGD) is a component of the multienzyme KGD complex catalyzing oxidative decarboxylation of a branch-point metabolite, namely α-ketoglutarate (KG). The overall reaction is thought to be a key controlling step in the Krebs cycle. In fact, the flux through the cycle correlates with the activity [1] and kinetic properties [2] of the complex. In its turn, KGD catalyzes the initial, irreversible and rate-limiting [3] stage of the overall process. Thus, effective specific inhibitors of KGD are of interest for the regulation of both the flux through the Krebs cycle and entry of glutamate to the cycle.

The mechanism of thiamine-dependent decarboxylation of α-ketoacids allows one to suppose inhibition of the reaction by a substrate analogue having a phosphonate group instead of α-carboxyl. The analogue binds to thiamine pyrophosphate (TPP) as a normal substrate but dephosphorylation does not take place according to the predictable properties of ketophosphonates [4]. This was shown to provide antimicrobial activity of phosphonate analogues of several amino acids and dipeptides (precursors of pyruvate) [5]. Inhibition of cell metabolism was observed after the transformation of these compounds to the corresponding pyruvate analogues, inhibiting the formation of acetylCoA from pyruvate both in cell extracts [5,6] and in the reaction catalyzed by the purified pyruvate dehydrogenase complex [7].

In this work the phosphonate analogue of KG, succinylphosphonate (SP), has been studied. Strong inhibition of KGD by SP has been shown, with methylated SP the less effective.

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2. EXPERIMENTAL

KGD was purified from pigeon breast muscle according to [8]. KGD activity was determined in the model reaction with ferricyanide in 0.05 M potassium phosphate, pH 6.3. The product accumulation (p_t) curves were analyzed as in [9]. SP excess was removed from KGD in 3–5 min by gel-centrifugation chromatography [10] using Sephadex G-50 fine in 0.05 M potassium phosphate, pH 6.3. SP and its methyl ether were synthesized according to [11]. Ferricyanide was obtained from Merck, KG from Serva and Sephadex G-50 fine from Pharmacia.

3. RESULTS

Fig. 1 shows the influence of SP on p_t curves of the KGD reaction. Comparing the curves 1–3 of Fig. 1 it is obvious that the effect of inhibitor depends on the time of its pre-incubation with KGD. No reaction to give products from SP was observed. This points to the absence of dephosphorylation since otherwise the normal reaction intermediate, hydroxybutyryl thiamine pyrophosphate, would be generated and oxidized in the course of the decarboxylation reaction.

As has been established earlier [12], the progress curves of the KGD reaction are biphasic (Fig. 1, curve 1) due to the kinetically slow transition of KGD during catalysis. The isomerisation is completed in several minutes leading to a decrease in both KGD activity and enzyme sensitivity to some effectors [9]. In fact, the structural analogues of KG inhibit the initial rate of KGD reaction (Fig. 2, A1) more than the rate reached after the isomerisation (A2). That is why they reduce the amplitude of the initial burst of activity (A1–A2)/A1 up to the complete disappearance of the burst. A similar

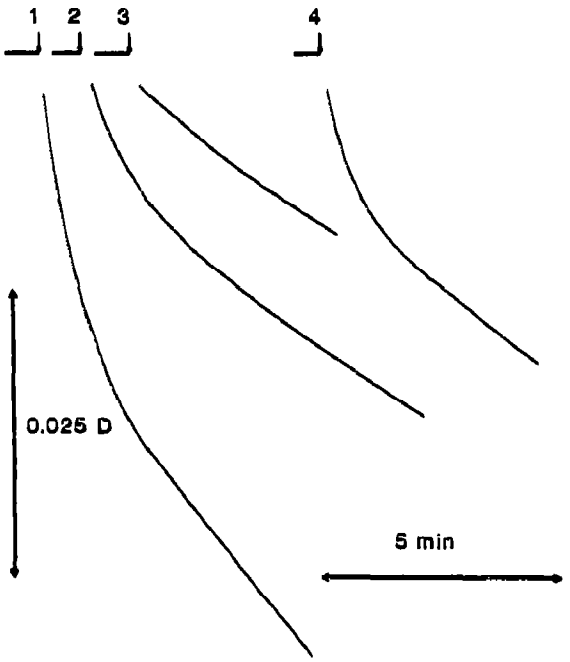

Fig. 1. The decrease in optical density at 420 nm in the course of the KGD reaction. KGD (0.011 mg/ml) was pre-incubated in the activity assay solution. The reaction was started (arrow) by simultaneous addition of KG (up to 0.5 mM) and ferricyanide (up to 0.6 mM) (curves 1–3) or only ferricyanide (curve 4). Curve 1, control (5 min in the buffer); curve 2, 5 s with 0.003 mM SP; curve 3, 5 min with 0.003 mM SP; curve 4, 5 min with 0.003 mM SP and 0.5 mM KG.

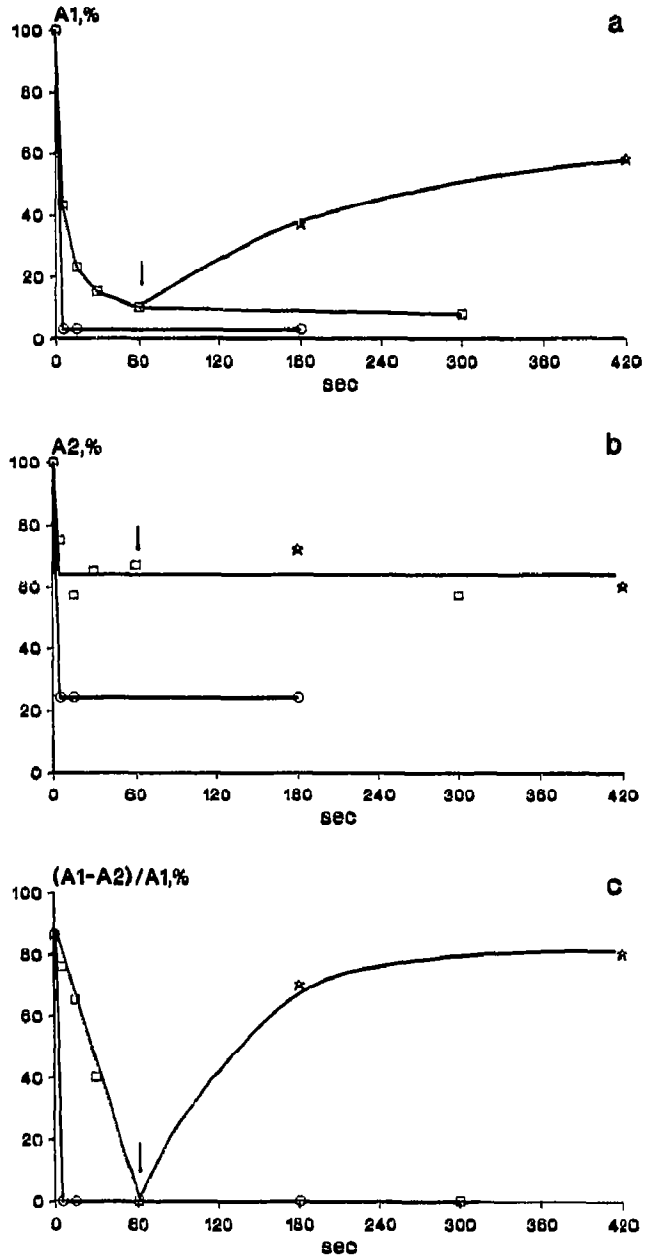

Fig. 2. Changes in the initial activity (a), the activity after the isomerisation (b) and the initial burst amplitude (c) during KGD pre-incubation with 0.003 mM SP (□), 0.019 mM SP (○) and after the addition of 0.5 mM KG to the medium with 0.003 mM SP and KGD (☆). The time of KG addition is shown by arrows.

effect is observed after the interaction of SP with KGD (Fig. 1). However, unlike other structural analogues of KG, SP influences the amplitude of the burst only after pre-incubation with the enzyme.

The rapid release of KGD from excess SP (see section 2) leads to the recovery of both the activity and biphasicity of the p_i curve. Thus, pre-incubation with the analogue does not cause the irreversible inactivation of KGD and the enzyme–inhibitor complex dissociates after the removal of SP.

Fig. 2 shows the time-dependence of KGD inhibition by different SP concentrations. At 0.003 mM SP, when the rate of the process is measurable, it is obvious that the inhibition of A1 (Fig. 2a) is slower, than that of A2 (Fig. 2b). In fact, A2 reaches the final level after some seconds of pre-incubation. However, the maximal inhibition of A1 requires about 1 min, as is seen also from Fig. 1 (curves 1–3).

The time dependence of A1 inhibition (Fig. 2a) correlates with the change in initial burst amplitude occurring at the same time (Fig. 2c), that is, the disappearance of the initial burst of activity (Fig. 1, curve 3), which causes more pronounced inhibition of A1 compared to A2 after pre-incubation with SP (Fig. 3a). Nevertheless, KGD inhibition by SP is not limited by the inhibition of initial activity burst only. In fact its amplitude is already zero after KGD pre-incubation with 0.003 mM SP, but raising the analogue concentration to 0.019 mM leads to a further inhibition which is not dependent on the change in the shape of the p_i curve (Fig. 2). KG added to the pre-incubation medium with KGD and SP prevents the time development of inhibition (Fig. 1). In this case the shape of the p_i curve does not essentially change even after 15–20 min of pre-incubation. Comparing curves 3 and 4 of Fig. 1 leads to a conclusion about the different effect of KG on the inhibition of A1

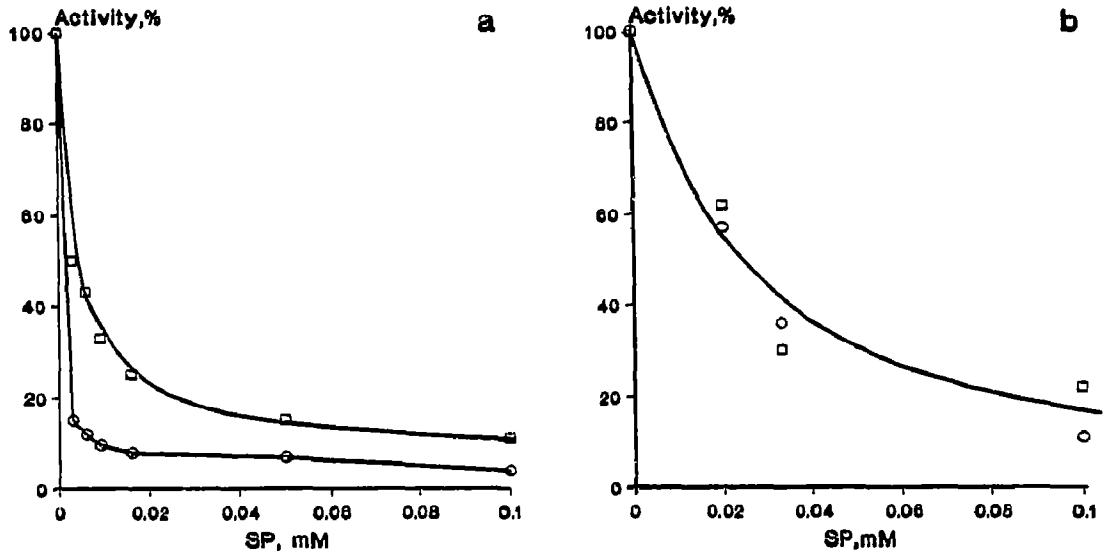

Fig. 3. The inhibition of KGD by SP after a 5 min pre-incubation with analogue (a) and without pre-incubation (b). (○) A1; (□) A2. KG concentration in the activity assay was 0.5 mM.

and A2. KG (0.5 mM) added to the pre-incubation medium with 0.003 mM SP completely prevents the inhibition caused by the disappearance of the initial burst of KGD activity. This provides a partial protection of A1 in the presence of KG. However, the inhibition of A2 (and proportional inhibition of A1) does not change.

The different effect of KG on the two types of KGD inhibition by SP is also revealed in another experiment in which KG is added to the enzyme already inhibited by SP (Fig. 2). The following incubation with 0.5 mM KG leads to the appearance of the initial burst of activity (Fig. 2c), causing an increase in A1 (Fig. 2a) without affecting A2 (Fig. 2b).

The protective effect of KG on the initial burst of KGD activity correlates with the difference observed in the inhibition of KGD pre-incubated (Fig. 3a) or not (Fig. 3b) with SP. In the latter case we see the proportional decrease in A1 and A2 which is characteristic of the inhibition in the presence of KG.

In order to reveal the role of different parts of the analogue molecule in the inhibition of KGD activity, we have studied the compound with a modified phosphonate group, namely the monomethyl ether of SP. The modification decreases the degree and rate of inhibition. In fact, the time necessary for the maximal inhibition is 5–6-times more when the methyl analogue (Fig. 4) is used instead of SP (Fig. 2). Besides, 0.019 mM SP causes 75% inhibition of A2 along with the complete disappearance of the initial burst (Fig. 2), whilst the

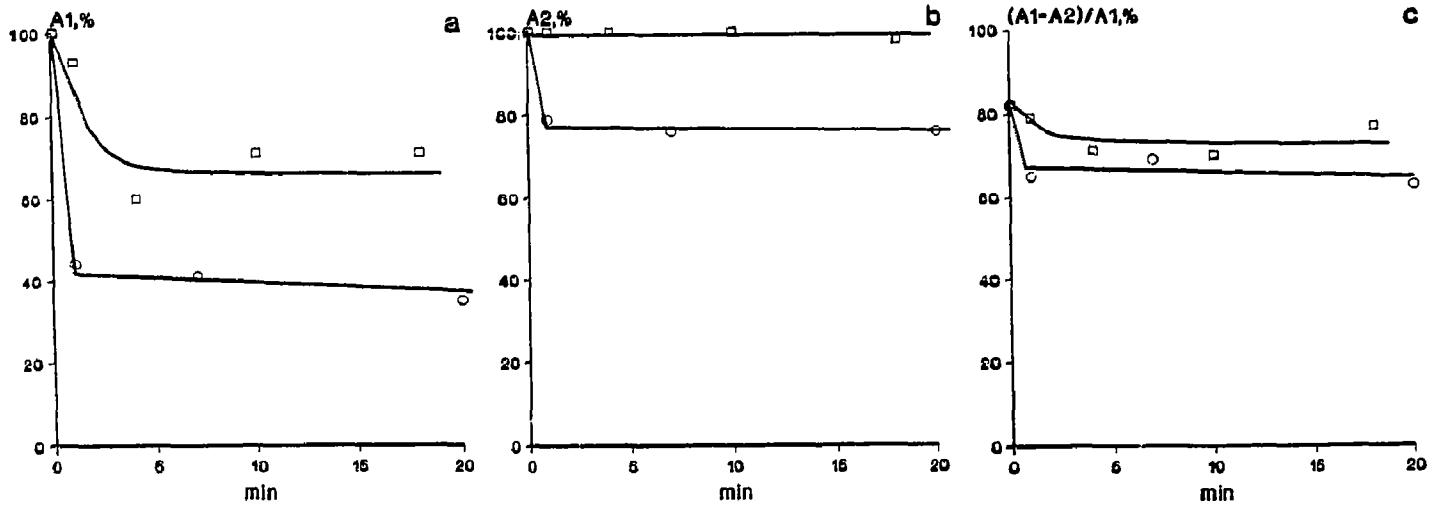

Fig. 4. Changes in the initial activity (a), the activity after the isomerisation (b) and the initial burst amplitude (c) during KGD pre-incubation with 0.003 mM (□) and 0.019 (○) mM methyl ether of SP.

methylated compound at the same concentration inhibits A2 only by 25% and decreases the burst amplitude by 15% (Fig. 4).

4. DISCUSSION

Among the structural analogues of KG whose inhibitory effect on KGD has been studied earlier [9] SP is the most powerful inhibitor of the enzyme. The inhibition by the phosphonate is strong enough even at 170-fold excess of KG (Figs. 1,2). This is consistent with the assumption that interaction of phosphonates with thiamine-dependent enzymes leads to the formation of transition state analogues [4].

Two stages of KGD inhibition by KG phosphonate analogues have been revealed. The proportional decrease of A1 and A2 without affecting the shape of the p_t curve is observed at the first stage. This type of inhibition is manifest when KG and SP act simultaneously, both in the pre-incubation medium (Figs. 1,2) and in the assay of KGD not pre-incubated with the analogue (Fig. 3b). The second stage is associated with a decrease in the initial burst of activity, causing an additional inhibition of A1 compared to A2.

According to the known mechanism of action of α-ketoacid phosphonate analogues they bind to the catalytic sites of thiamin enzymes, interacting with the C2 atom of TPP [4]. Blocking the catalytic center of KGD by the phosphonates appears to occur at the first stage of the inhibition and is complete in a few seconds. The high rate of this stage is comparable to that of enzyme–substrate complex formation. Moreover, the proportional inhibition of A1 and A2 observed at this stage also supports such a mechanism. Really, the formation of the covalent bond between the analogue and TPP, which is tightly bound in the KGD active site, must provide the stability of the enzyme–inhibitor complex relatively independent of the enzyme's conformational state. This is in good accord with the fact that both the initial KGD (with the activity A1) and the enzyme isomerized during the reaction (having the activity A2) are inhibited at this stage to the same extent.

The necessity of pre-incubation with the phosphonates in order to inhibit the initial burst of KGD activity supports the earlier assumption that it is conditioned by the kinetically slow transition of KGD [12]. By interacting with the catalytic site of KGD SP seems to induce a conformational change similar to that observed in the course of the KGD reaction. The enzyme changed during pre-incubation with the analogue does not isomerize in the following reaction with KG. This results in linear p_t curves after the pre-incubation with SP (Fig. 1, curve 3).

A change in the structure of the initial enzyme–inhibitor complex which increased the inhibitory effect was observed also when the phosphonate analogue of pyruvate interacted with pyruvate dehydrogenase [7]. Thus both KGD and pyruvate dehydrogenase isomerize slowly when bound to phosphonate analogues of substrates.

When KG is present in the pre-incubation medium with SP (Figs. 1,2) we can see the effect on the enzyme of both SP (the inhibition of A2) and KG (the protection from isomerisation of the enzyme–inhibitor complex). Thus, SP and KG are simultaneously bound to KGD.

There are two possibilities for such a binding. One involves the half of the sites reactivity of KGD dimer to SP. KG could bind to the active site of the second dimer subunit, preventing isomerisation of the enzyme–inhibitor complex. Considering this scheme, we must assume that SP bound to one subunit inhibits the catalysis in another, because there is more than 50% inhibition by SP in the presence of KG (Fig. 3b). At the same time, SP does not prevent KG binding, which is manifested in the protection of the initial burst. If KG were bound to TPP of the second dimer subunit why could it not be decarboxylated? This contradiction points to another possibility, that KG preventing the isomerisation of enzyme–inhibitor complex is bound to the non-catalytic site. Such a supposition is in accordance with our earlier investigation [9] favoring the existence of the second KG binding site per KGD subunit.

If KG in the non-catalytic site prevents the isomerisation of the transition state analogue (Fig. 1), it could similarly protect the transition state of the normal enzymatic reaction. This could explain the dependence of the shape of the p_t curves when the KG concentration increases [9]. At low substrate concentration the isomerisation rate is too high to be detectable in the steady-state of the KGD reaction. Thus there is no significant biphasicity in the time-course of the reaction in this case [9]. The biphasicity is substantial, however, in the medium with high substrate concentration. Obviously, then, the second KG binding site is saturated and the transition becomes slow enough to be observed as the fast stage of KGD inactivation during the reaction.

There is no significant change in the p_t curve when KGD is pre-incubated with SP and KG for 15–20 min. However, in the course of the KGD reaction the isomerisation is completed in 5 min (Fig. 1). Thus KG is less effective in the last case. Because the difference between the enzyme–substrate and the enzyme–inhibitor complexes lies in the substitution of KG α-carboxyl by the phosphonate group, the isomerisation seems to be due to the α-carboxyl transformation in the KGD active site. This is supported by the experiments with methylated SP, which is far less effective in producing the isomerisation (Fig. 4c) than SP (Fig. 2c). Thus the more distant the structure is from α-carboxyl the less pronounced is the KGD isomerisation. This could argue for the existence of physiologically important regulation of KGD by CO_2, involving slow conformational change of the enzyme decreasing its activity.

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